The physiological roles of ER stress transducer BBF2H7/CREB3L2 and its potential as a target of disease therapy

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Abstract

BBF2H7 is an endoplasmic reticulum (ER)-resident transmembrane transcription factor, that is cleaved at the transmembrane region in response to physiological and pathophysiological ER stress to generate two fragments; the cytoplasmic N-terminal fragment containing transcription activation and basic leucine zipper (bZIP) domains translocates into the nucleus to act as a transcription factor. Conversely, the luminal C-terminal fragment is extracellularly secreted and promotes the proliferation of neighboring cells via activation of hedgehog (Hh) signaling. In developing cartilage, the dual N- and C-terminal functions of BBF2H7 enable chondrocytes to simultaneously orchestrate distinct cellular events for differentiation and proliferation via the activation of the secretory pathway by the N-terminus and the Hh signaling by the C-terminus. Interestingly, the secreted BBF2H7 C-terminus is involved in cancer cell proliferation by the activation of Hh signaling, which is well known to facilitate tumorigenesis. In this review, we summarize the biological roles of BBF2H7 in developing cartilage and cancer cells, and discuss the potential of BBF2H7 as a novel target for cancer therapy.

Keywords: BBF2H7, endoplasmic reticulum stress, unfolded protein response, chondrogenesis, hedgehog signaling, cell proliferation, cancer treatment

1. Introduction

The endoplasmic reticulum (ER) is the central cellular organelle providing a specialized environment for the synthesis, folding and post-translational modifications of secretory and membrane proteins. Under various pathophysiological conditions including ER calcium depletion, oxidative stress, hypoglycemia, expression of mutant proteins and hypoxia, unfolded or misfolded proteins accumulate in the ER lumen. These conditions are collectively termed ER stress (Kaufman, 2002; Ron, 2002). Upon ER stress, cells sense the unfolded proteins, subsequently the activation of an integral signal transduction called the unfolded protein response (UPR) (Kaufman, 2002; Ron, 2002). Activated UPR leads to transient translational attenuation, followed by transcriptional induction of ER-resident chaperone capacity and degradation of accumulated unfolded proteins in the ER (ER-associated degradation, ERAD) to avoid cellular damage and apoptotic cell death (Kaufman, 2002; Ron, 2002).

In mammalian cells, three major and ubiquitously expressed ER stress transducers have been well established: PKR-like endoplasmic reticulum kinase (PERK) (Harding, Zhang, & Ron, 1999), inositol-requiring enzyme 1 (IRE1) (Tirasophon, Welihinda, & Kaufman, 1998), and activating transcription factor 6 (ATF6) (Yoshida et al., 2000). These molecules are localized at the ER membrane, and are activated in response to ER stress. The signaling pathways and the physiological functions of these canonical ER stress transducers have been detailed elsewhere (Ron & Walter, 2007).

In addition to the three canonical ER stress transducers, novel types of ER stress transducers that share domains of high sequence similarity with ATF6 have been identified. These ER stress transducers include BBF2H7/CREB3L2 (Kondo et al., 2007; Saito et al., 2009), OASIS/CREB3L1 (Kondo et al., 2005; Murakami et al., 2009), Luman/LZIP/CREB3 (DenBoer et al., 2005; Lu, Yang, O'Hare, & Misra, 1997), CREBH/CREB3L3 (Zhang et al., 2006), and CREB4/AIbZIP/Tisp40/CREB3L4 (Nagamori et al., 2005; Stirling & O'Hare,

2006). These proteins have a transmembrane domain, which allows them to localize at the ER membrane, a transcription activation domain and basic leucine zipper (bZIP) domain. The bZIP domains are extremely well conserved within these members. An additional region of ~30 residues is located adjacent to the N-terminal end of the bZIP region, and is conserved in the OASIS family members, but not ATF6. As this region is not a conserved site of the typical consensus bZIP DNA binding domain, distinct and particular roles may be provided in this unique sequence. Furthermore, each protein commonly contains the consensus sequence for cleavage by site-1 protease (S1P) and site-2 protease (S2P) at the transmembrane region, indicating that they are processed by regulated intramembrane proteolysis (RIP), like ATF6 (Bailey & O'Hare, 2007).

OASIS family proteins (except Luman) show unique cell or tissue-specific expression patterns. BBF2H7, OASIS, CREBH and AIbZIP are preferentially expressed in chondrocytes (Saito et al., 2009), osteoblasts and astrocytes (Kondo et al., 2005; Murakami et al., 2009), liver cells (Omori et al., 2001), and prostate and testis (Cao et al., 2002), respectively. Thus, these transcription factors may be involved in distinct physiological responses that are dependent on the cells or tissues. Indeed, recent studies indicate that OASIS family members are specialized in biological regulation including cell differentiation, maturation, and maintenance of basal cellular homeostasis rather than dealing with unfolded proteins: BBF2H7 in secretion of matrix proteins and chondrocyte differentiation (Saito et al., 2009); OASIS in osteogenesis and maturation of osteoblast, astrocyte and goblet cell (Asada et al., 2012; Murakami et al., 2009; Saito et al., 2012); Luman in osteoclastogenesis and dendritic cell differentiation (Eleveld-Trancikova et al., 2010; Kanemoto et al., 2015); CREBH in the acute phase response in the liver (Zhang et al., 2006); and AIbZIP in spermatogenesis (Adham et al., 2005; Yamaguchi et al., 1999). In this review, we focus on one of the OASIS family members, BBF2H7, and describe its biological functions.

2. Structure, distribution and activation mechanism of BBF2H7

The *Bbf2h7* gene was first identified by virtue of its involvement in the gene translocation of a fusion event between the *fused in sarcoma* (*FUS*) gene on chromosome 16 and *Bbf2h7* gene on chromosome 7 (Storlazzi et al., 2003). BBF2H7 is a transmembrane bZIP transcription factor (Figure 1A) and is a notably unstable protein that is easily degraded via the ubiquitin-proteasome pathway under normal conditions (Kondo et al., 2012). HMG-CoA reductase degradation 1 (HRD1), an ER-resident E3 ubiquitin ligase, ubiquitinates BBF2H7. Alternatively, ER stress conditions dissociate the interaction between HRD1 and BBF2H7, resulting in the enhancement of BBF2H7 stability. In response to ER stress, stabilized BBF2H7 is cleaved at the transmembrane region by RIP to generate a cytoplasmic N-terminus, which contains a transcription activation and bZIP domains, and luminal C-terminus (Figure 1B). The cleaved N-terminal fragment translocates into the nucleus and binds to the cyclic AMP-response element (CRE) sequence to activate transcription of target genes (Kondo et al., 2007).

Although BBF2H7 is widely expressed in many tissues and organs, the most intense signals are observed in the proliferating zone of the developing cartilage (Figure 1C) (Saito et al., 2009). In developing cartilage, the transcription of *Bbf2h7* is regulated by Sex determining region Y-related high-mobility group box 9 (Sox9) through the binding of Sox9 to the Sox DNA-binding motif in the *Bbf2h7* promoter (Hino et al., 2014). Sox9 is a crucial factor for chondrocyte differentiation, and facilitates the expression of one of the major cartilage matrix proteins type II collagen (Col II) (Akiyama, Chaboissier, Martin, Schedl, & de Crombrugghe, 2002). It is known that Sox9 plays pivotal roles not only in chondrocyte differentiation but also in glial differentiation (Stolt et al., 2003). The number of glial cells differentiated from neural precursor cells (NPCs) is severely decreased in mice specifically ablating Sox9 expression in NPCs by Cre/loxP recombination system (Stolt et al., 2003).

However, the expression of BBF2H7 is not affected by Sox9 in primary cultured NPCs unlike that of chondrocytes (Hino et al., 2014). Although the precise reason for the different regulatory mechanisms of BBF2H7 expression between chondrocytes and NPCs is unclear, it may be explained by the following possibilities. 1) The expression of *Bbf2h7* may be controlled by epigenetic regulation such as the methylation and acetylation status of the *Bbf2h7* promoter region and histone modifications. 2) Specific cofactors may be necessary for transcriptional activation of *Bbf2h7* by Sox9. Further studies are necessary to elucidate the molecular mechanisms for selective transcriptional activation of *Bbf2h7* in each cell and tissue type.

3. Secretion of extracellular matrix proteins promoted by BBF2H7 N-terminus in chondrocyte differentiation

Bbf2h7-deficient mice exhibit severe chondrodysplasia involving short limbs, a protruding tongue and a distended belly and die by suffocation shortly after birth because of an immature chest cavity (Saito et al., 2009). In developing cartilage of *Bbf2h7*-deficient mice, the typical columnar structure is lacking in the proliferating zone, and the size of the hypertrophic zone is decreased involving a significant reduction of extracellular matrix (ECM) proteins in the extracellular space (Saito et al., 2009). Proliferating chondrocytes show a significant decrease in the number of cells, and abnormally expanded rough ER containing aggregated cartilage matrix proteins including Col II and cartilage oligomeric matrix protein (COMP) (Saito et al., 2009). These observation indicate that secretion of ECM proteins is prevented in the proliferating zone of *Bbf2h7*-deficient mice, and the impaired protein secretion leads to the disruption of the ECM network and cartilage zone formation.

In an analysis of gene expression in primary cultured chondrocytes, *Sec23a* was identified as one of the targets for BBF2H7 (Saito et al., 2009). The BBF2H7 N-terminus directly binds to CRE sequence in the promoter region of *Sec23a*. Sec23a recruits other components of the COPII vesicle, including Sec13/31 to form a

complete complex before transporting secretory proteins from the ER to the Golgi (Fromme, Orci, & Schekman, 2008; Paccaud et al., 1996). For chondrocytes to secrete large amounts of cartilage ECM proteins, it is necessary to smoothly transport secretory materials from the ER to either the Golgi or cellular membrane. It is inevitable that such functions will be acquired during the process of growing from undifferentiated cells into mature chondrocytes, and activation of the BBF2H7-Sec23a pathway is essential for developing a series of the secretory machinery.

Importantly, ER stress is necessary for the activation of BBF2H7. When and in what manner does ER stress occur in chondrocytes? During differentiation, chondrocytes secrete abundant cartilage matrix proteins. Simultaneously, ER stress markers *Bip*, *Pdi*, *Grp94* and *Edem* are slightly upregulated corresponding with the secretion (Saito et al., 2009), indicating that physiological ER stress is induced in chondrocytes during normal differentiation. Chondroprogenitor cells synthesize relatively low levels of matrix proteins. On differentiation into chondrocytes, abundant cartilage matrix proteins are produced in ER lumen. These exponential syntheses of secretory proteins increase the burden on the ER, leading to the induction of physiological ER stress and the cleavage of BBF2H7. Similar phenomena are also seen in plasma cell differentiation (Iwakoshi et al., 2003; Reimold et al., 2001). For the full development of antibody secretion, the UPR signaling activated by ER stress is essential. The downstream molecule of UPR signaling, x-box binding protein 1 (XBP1), is required for the differentiation into plasma cells, which synthesize abundant immunoglobulin (Iwakoshi et al., 2003; Reimold et al., 2001). *Xbp1*-deficient lymphocytes possess normal number of activated B lymphocytes. However, they are unable to secrete immunoglobulin of any isotype, because of the absence of plasma cells (Reimold et al., 2001). Therefore, activation of the UPR by ER stress is necessary for the maturation of immature cells to professional secretory cells. Taken together, the activation of BBF2H7 depends on the physiological ER stress caused by the augmented production

of cartilage matrix proteins, eventually enabling chondrocytes to acquire the well-developed secretory machinery as professional secretory cells.

4. Chondrocyte proliferation regulated by secreted BBF2H7 C-terminus via activation of hedgehog signaling

In contrast to the domain structure of BBF2H7 N-terminus, the ER luminal C-terminal domain of BBF2H7 has no recognized functional domains, and the biological functions were unexplored. Additionally, the cause for the significant decrease in the number of *Bbf2h7*-deficient chondrocytes was unclear. Recent studies have shown that the BBF2H7 C-terminus processed in response to physiological ER stress is secreted from chondrocytes into the extracellular space as a signaling molecule. The secreted C-terminus interacts with Indian hedgehog (Ihh) and Patched-1 (Ptch1) of neighboring chondrocytes to promote chondrocyte proliferation via the activation of hedgehog (Hh) signaling (Figure 2) (Saito et al., 2014).

Ihh, which is strongly expressed in prehypertrophic chondrocytes, is diffused to the periarticular region (Chung, Schipani, McMahon, & Kronenberg, 2001). Ihh reached to periarticular region binds to Ptch1, subsequently inducing parathyroid hormone-related protein (PTHrP) expression (Vortkamp et al., 1996). PTHrP promotes chondrocyte proliferation and inhibits differentiation from proliferating to hypertrophic chondrocytes through binding to its receptor, parathyroid hormone 1 receptor (Deckelbaum, Chan, Miao, Goltzman, & Karaplis, 2002). Once diffused PTHrP from periarticular region cannot reach to the distal site of proliferating chondrocytes caused by the sufficient extension of the proliferating zone, the differentiation from proliferating to hypertrophic chondrocytes is accelerated to organize the hypertrophic zone. Thus, Hh signaling activated by Ihh simultaneously regulates chondrocyte proliferation and differentiation, consequently fine-tuning the length of the proliferating zone in developing cartilage (Kronenberg, 2003). The secreted BBF2H7 C-terminus is unable

to act as an Hh ligand. However, this fragment binds to both the Hh ligand Ihh and its receptor Ptch1 (Saito et al., 2014). The BBF2H7 C-terminus secreted from proliferating chondrocytes traps Ihh released from prehypertrophic chondrocytes. The C-terminus-Ihh complex then efficiently binds to Ptch1 at the cell surface of the periarticular region. Hence, BBF2H7 C-terminus plays a crucial role in delivering Ihh to the periarticular region remote from prehypertrophic zone to form ligand-receptor complex, thereby sufficiently activating the Ihh-PTHrP pathway and chondrocyte proliferation over the distance. In the context, BBF2H7 controls two essential machineries for chondrogenesis; the cleaved N-terminus promotes the secretion of cartilage matrix proteins via Sec23a induction. Simultaneously, the C-terminus regulates proliferation of proliferating chondrocytes and differentiation from proliferating to hypertrophic chondrocytes via activation of Hh signaling. These dual functions of BBF2H7 may contribute to the substantial growth of developing cartilage.

5. Cancer cell proliferation promoted by Hh signaling and secreted BBF2H7 C-terminus via activation of Hh signaling

5.1 Hh signaling and cancer

Hh signaling modulates diverse events including cell proliferation, differentiation, tissue patterning and formation during embryonic stage (Varjosalo & Taipale, 2008). In adult stage, Hh signaling maintains stem cell pluripotency and regulates its proliferation in several tissues including the hematopoietic system (Bhardwaj et al., 2001), mammary gland (S. Liu et al., 2006) and nervous system (Ahn & Joyner, 2005). In mammalian cells, Ihh, Sonic hedgehog (Shh) and Desert hedgehog are well known as Hh ligands (Ingham & McMahon, 2001). These ligands bind to the transmembrane Hh receptor, Ptch1. Another transmembrane protein, Smoothened (Smo), is negatively regulated by Ptch1 in the absence of Hh ligands (Denef, Neubuser, Perez, & Cohen, 2000). Once Hh ligands bind to Ptch1, Smo is relieved from the inhibition by Ptch1, and activates the transcription factor

glioma-associated oncogene 1 (Gli1) (Denef et al., 2000; Ruiz i Altaba, 1998). Activated Gli1 then promotes the expression of target genes such as *Gli1*, *Ptch1*, *Forkhead boxl1*, *Cyclin D1* and *Cyclin E1* (Duman-Scheel, Weng, Xin, & Du, 2002; Katoh & Katoh, 2009).

Recent studies showed that uncontrolled activation of Hh signaling leads to survival of cancer cells and tumor formation (Berman et al., 2002; Clement, Sanchez, de Tribolet, Radovanovic, & Ruiz i Altaba, 2007; Dierks et al., 2008; Kubo et al., 2004; Sanchez et al., 2004). Gli1 upregulation, followed by the activation of Hh signaling is associated with the growth of glioblastoma (Dahmane et al., 2001). *Gli1* was identified as an amplified gene in glioblastoma (Kinzler et al., 1987). The upregulation of Gli1 contributes to enhanced proliferation of glioblastoma through the activation of Hh signaling (Dahmane et al., 2001). The uncontrolled secretion of Hh ligands accelerates cancer cell proliferation and tumor growth in an autocrine and paracrine manner. Overproduction of Hh ligands is observed in pancreatic cancer, breast cancer, prostate cancer and lung adenocarcinoma (Kim et al., 2013; Oro et al., 1997; Pasca di Magliano & Hebrok, 2003; Scales & de Sauvage, 2009; Tao, Mao, Zhang, & Li, 2011). Additionally, loss of function in *Ptch1* and an active mutant of *Smo* trigger to aberrant activation of Hh signaling. Patients with Gorlin syndrome, also known as basal cell nevus syndrome, have inherited an inactivating mutation in *Ptch1* gene, leading to constitutively active Hh signaling in the absence of Hh ligands (Johnson et al., 1996). These patients easily develop basal cell carcinomas (BCC), a skin tumor of keratinocytes, medulloblastomas and rhabdomyosarcomas (Johnson et al., 1996). The constitutively active mutant of *Smo* is also detected in BCC (Xie et al., 1998). The active mutant shows diminished inhibition by Ptch1, resulting in the overactivation of Hh signaling.

During tumor progression, uncontrolled activation of Hh signaling promotes cancer metastasis (Flemban & Qualtrough, 2015; Hanna & Shevde, 2016). Activated Gli1 induces migratory capacity via induction of Snail, a regulator of

epithelial-mesenchymal transition. Upregulation of Snail by Gli1 leads to loss of cell-cell adhesion and the production of ECM-degrading enzymes. (Louro et al., 2002). Taken together, uncontrolled activation of Hh signaling is closely involved in various tumor growth and metastasis.

5.2 Acceleration of cancer cell proliferation by the secreted BBF2H7 C-terminus

Interestingly, BBF2H7, which promotes chondrocyte proliferation by its C-terminal fragments through the activation of Hh signaling, is involved in cancer cell proliferation and tumor growth (Iwamoto et al., 2015). *Bbf2h7* was originally identified as 3'-partner of *FUS* in the fusion gene found in low-grade fibromyxoid sarcoma, which is observed overactivation of Hh signaling (Storlazzi et al., 2003). The FUS-BBF2H7 fusion protein contains the N-terminus of FUS, and the bZIP domain, transmembrane domain, and full C-terminal luminal domain of BBF2H7. Additionally, recent studies have shown overexpression of BBF2H7 in human glioblastoma (Sheng et al., 2010), impregnating that BBF2H7 contributes to tumor growth through the C-terminus-mediated activation of Hh signaling. Indeed, secretion of BBF2H7 C-terminal fragments is observed in glioblastoma, breast cancer, cervical cancer and prostate cancer cell lines (Iwamoto et al., 2015). These cells show activation of Hh signaling, and the proliferation is accelerated in an Hh ligand- and BBF2H7 C-terminus-dependent manner. The knockdown of *Bbf2h7* in glioblastoma cell line suppresses its proliferation (Iwamoto et al., 2015), strongly suggesting that the secreted BBF2H7 C-terminus may promote cancer cell proliferation and tumor growth via the activation of Hh signaling.

Bbf2h7 mRNA is highly expressed in several cancers. However, the mechanism for the transcriptional regulation of *Bbf2h7* in cancer cells is still unclear. One possibility is Sox9, which directly induces expression of *Bbf2h7* in chondrocytes (Hino et al., 2014), may induce the expression of *Bbf2h7* in cancer cells. Sox9 is highly expressed and accelerates tumorigenicity in prostate cancer,

colon cancer and glioblastoma (Pritchett, Athwal, Roberts, Hanley, & Hanley, 2011). Alternatively, knockdown of *Sox9* by siRNA reduced cell proliferation in glioma cell lines (L. Wang et al., 2012), implying a relationship among Sox9, BBF2H7 and tumor growth. Although further investigation is necessary, it is possible that Sox9 is involved in the proliferation of cancer cells through the induction of *Bbf2h7* expression. Another question regards the mechanism for the activation of BBF2H7 in cancer cells. The tumor microenvironment is characterized by hypoxia, nutrient deprivation and low pH (Brown & Giaccia, 1998). These environmental factors can trigger ER dysfunctions and the induction of ER stress (M. Wang & Kaufman, 2014). Several studies reported that UPR signaling is activated in tumor tissues. The activation of UPR signaling allows cancer cells to adapt to tumor microenvironment, resulting in survival of these cells (Hetz, Chevet, & Harding, 2013; S. Wang & Kaufman, 2012). The specific conditions of the tumor microenvironment, which is appropriate for the induction of ER stress, may promote ER stress-induced cleavage of BBF2H7 and the secretion of the C-terminus.

6. Approaches targeting Hh signaling components for cancer treatment

Hh signaling is one of the most attractive targets for the development of anti-cancer drugs, since the aberrant activation of this signaling affects critical processes in tumor growth including proliferation, invasion and metastasis of cancer cells (Hanna & Shevde, 2016; Rimkus, Carpenter, Qasem, Chan, & Lo, 2016). Actually, various components of the Hh pathway are the focus of attention as therapeutic targets.

6.1 Hh ligands

Ligand-dependent activation of Hh signaling is involved in cancer cell proliferation and tumorigenesis (Pasca di Magliano & Hebrok, 2003; Scales & de Sauvage, 2009). Thus, Hh ligands are reasonable targets against Hh signaling-dependent cancer. Hh ligands function in most upstream of Hh signaling as

initial factors, indicating that anti-cancer drugs targeting Hh ligands may broadly shut-down Hh signaling. Of importance, these drugs are easily accessible to the secreted targets in extracellular space, and they do not need to be incorporated into cells. Beneficial therapeutic effects against several cancer overexpressing Hh ligands can be expected by utilizing anti-Shh neutralizing antibody. The intraperitoneally administrated Shh monoclonal antibody 5E1 inhibits the growth of medulloblastoma and pancreatic cancer in a mouse model (Chang, Foltz, Chaudary, Hill, & Hedley, 2013; Coon et al., 2010).

6.2 Smo and Ptch1

Smo inhibitors prevent the activation of Gli1, followed by suppressing the induction of its target genes that are associated with tumor growth and progression (Gonnissen, Isebaert, & Haustermans, 2015; Rimkus et al., 2016). Vismodegib, a second generation Smo inhibitor, was approved by the Food and Drug Administration (FDA) as the first anti-cancer drug targeting the Shh pathway in several tumors (Robarge et al., 2009; Sekulic et al., 2012). Although there is some issues with *Smo* mutants exhibiting vismodegib-resistance (Metcalfe & de Sauvage, 2011), vismodegib may have exceptional prospects as a therapeutic approach against Hh signaling-dependent tumors. Ptch1 is another cell surface target for the development of anti-cancer drugs. A previous study showed that an anti-Ptch1 antibody suppresses proliferation of pancreatic cancer cells through the inhibition of Hh signaling (Nakamura et al., 2007).

6.3 Gli1

Gli1 is a most downstream molecule of Hh signaling, and directly regulates the induction of various Hh target genes that are related to tumor growth. The inhibition of Gli1 may effectively suppress the growth of various tumors activating Hh signaling, since almost all signals stimulated by Hh ligands converge to Gli1 activation. The Gli1 inhibitor GANT-61 binds to Gli1 and robustly inhibits the growth of tumors including rhabdomyosarcoma, osteo-sarcoma, neuroblastoma and ovarian cancer

through the attenuation of Gli1-mediated gene induction (Chen et al., 2014; Lauth, Bergstrom, Shimokawa, & Toftgard, 2007; Shahi, Holt, & Rebhun, 2014; Srivastava et al., 2014; Wickstrom et al., 2013).

Although it is necessary to precisely determine the aberrant component responsible for enhancing the growth in each tumor, the components of Hh signaling are prospective targets for therapeutic strategies against various cancers, and several anti-cancer drugs targeting these components are achieving in clinical trials.

7. Potential of BBF2H7 C-terminus as a target for cancer therapy

BBF2H7, modulating Hh signaling in cancer cells, also has potential as a new target for cancer treatment. Anti-BBF2H7 C-terminus specific antibodies may interfere with the function of the C-terminal fragment to act as neutralizing antibodies (Figure 3). Antibodies recognizing Hh ligand- or the Hh receptor-binding domain of BBF2H7 C-terminus may inhibit the activation of Hh signaling mediated by the C-terminus. BBF2H7 C-terminus promotes to form ligand-receptor complexes, which is first event for activating Hh signaling in extracellular space. Therefore, the neutralizing antibodies against the C-terminus may easily reach to the secreted C-terminus, since the antibodies do not have to be incorporated into the cells. Additionally, the neutralizing antibodies can block the first step of Hh signaling, and may effectively inhibit diverse Hh signaling just like anti-Hh ligand neutralizing antibodies. Furthermore, the BBF2H7 C-terminus binds to all three Hh ligands (Saito et al., 2014), indicating that anti-BBF2H7 C-terminus neutralizing antibodies may suppress cancer cell proliferation mediated by all three Hh ligands. Hence, a therapeutic strategy targeting the secreted BBF2H7 C-terminus has the potential to comprehensively suppress the uncontrolled activation of Hh signaling in various tumors.

Inhibition of UPR is another strategy to prevent the secretion of BBF2H7 C-terminus in tumor tissues. However, a recent study reported that the constitutively activated oncogenic *Smo* mutant is selectively degraded in the ERAD pathway

which is activated in the downstream of UPR signaling (Marada, Stewart, Bodeen, Han, & Ogden, 2013). It is possible that UPR suppression may promote rather than inhibit cancer cell proliferation in some tumors. Thus, the application of a neutralizing antibody is a more practical strategy compared with that of UPR inhibition.

BBF2H7 is induced in glioblastoma, invasive ductal breast carcinoma, cervical squamous carcinoma, prostate adenocarcinoma and colon adenocarcinoma (Iwamoto et al., 2015). However, the expression of BBF2H7 is unchanged in gastric adenocarcinoma and pancreatic adenocarcinoma, even though their cell proliferation is promoted by activation of Hh signaling (Pasca di Magliano & Hebrok, 2003; Scales & de Sauvage, 2009). Although further studies are required, other membrane-associated proteins such as biregional Cdon-binding protein (Boc), cell-adhesion molecule-related/down-regulated by oncogenes (Cdon) and growth arrest-specific 1 (Gas1) (Y. Liu, May, & Fan, 2001; Tenzen et al., 2006), which are not Hh ligands, but can act as Hh cofactors like BBF2H7 C-terminus, may affect Hh signaling instead of BBF2H7 C-terminus in these cancer cells. It is important to determine the expression of BBF2H7 and other cofactors in Hh signaling-activated tumors to ensure sufficient effect for blocking tumor growth by anti-BBF2H7 C-terminus neutralizing antibody.

8. Conclusion

Physiologically, the dual N- and C-terminal functions of BBF2H7 simultaneously regulate distinct cellular events in developing cartilage; accelerating the secretion of cartilage matrix proteins by the N-terminus, and promoting chondrocyte proliferation by the C-terminus. Pathophysiologically, the induction of ER stress derived from the tumor microenvironment and the subsequent cleavage of BBF2H7 triggers the secretion of its C-terminus, resulting in the promotion of cancer cell proliferation through the activation of Hh signaling during tumorigenesis. BBF2H7 is a key regulator of cell proliferation in Hh-dependent cancers, and may provide unconventional

perspectives to understand the link between the mechanism for tumor growth and signal transduction derived from ER. In addition, the secreted BBF2H7 C-terminal fragment may be a novel therapeutic target for blocking the activation of Hh signaling, potentially providing breakthroughs for strategies of cancer therapy.

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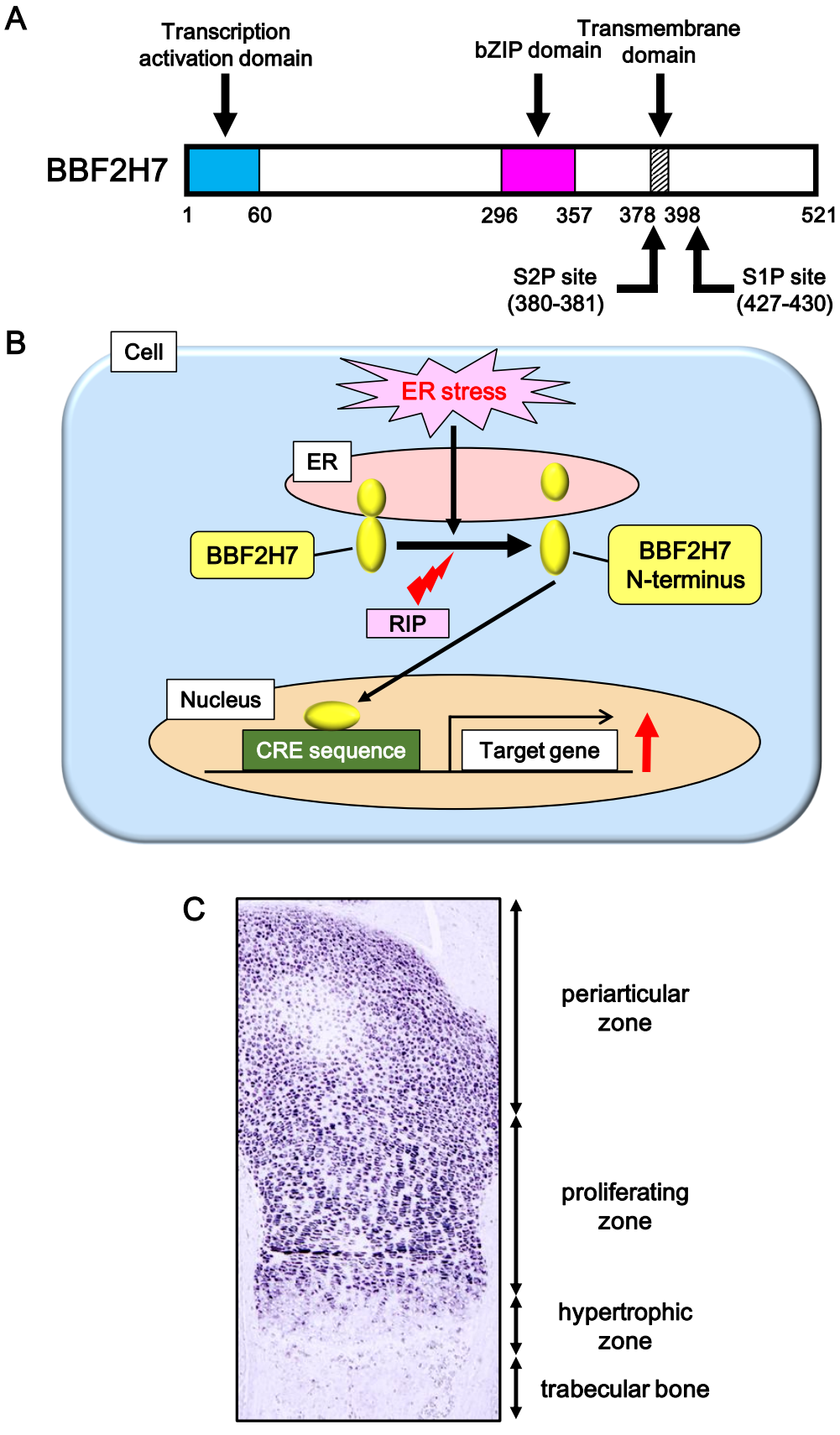

Figure 1

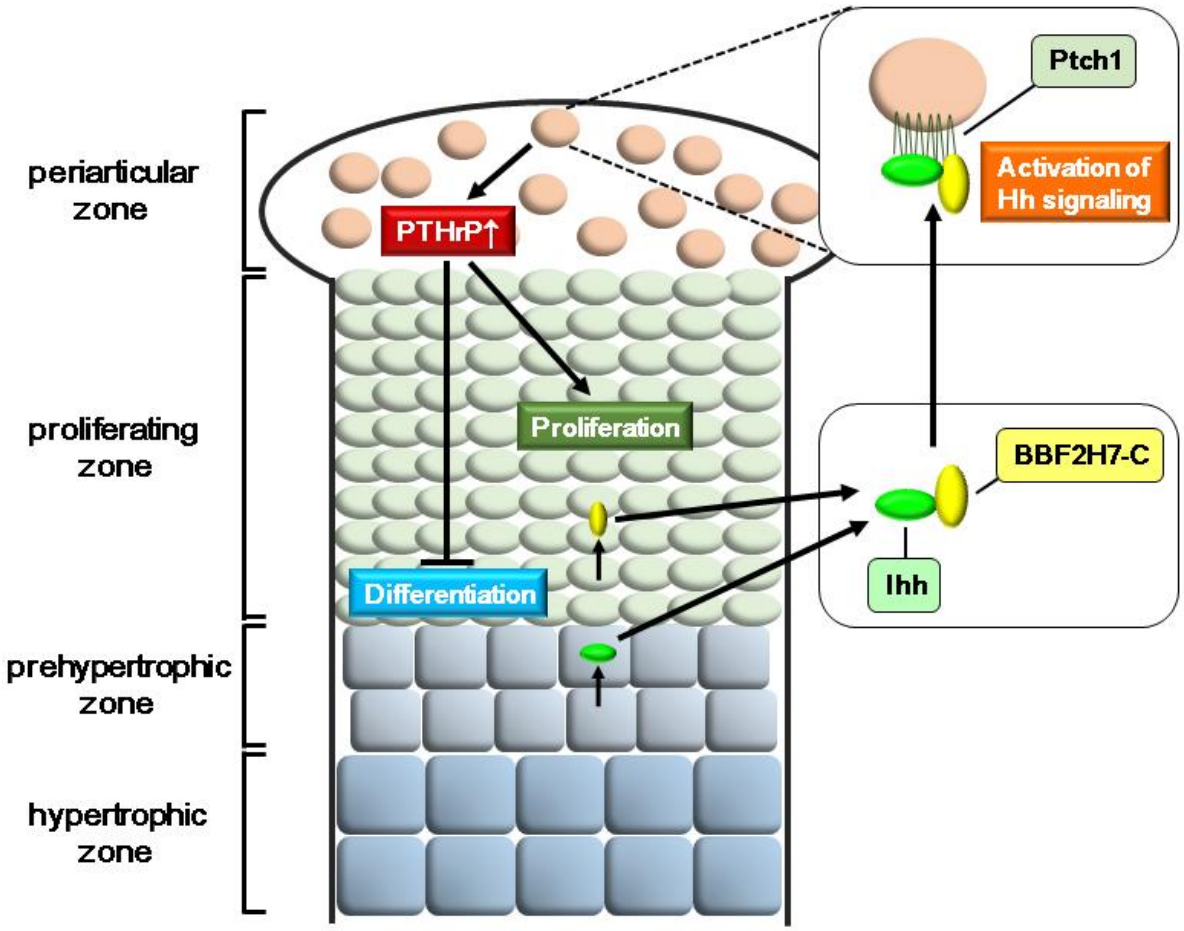

Figure 2

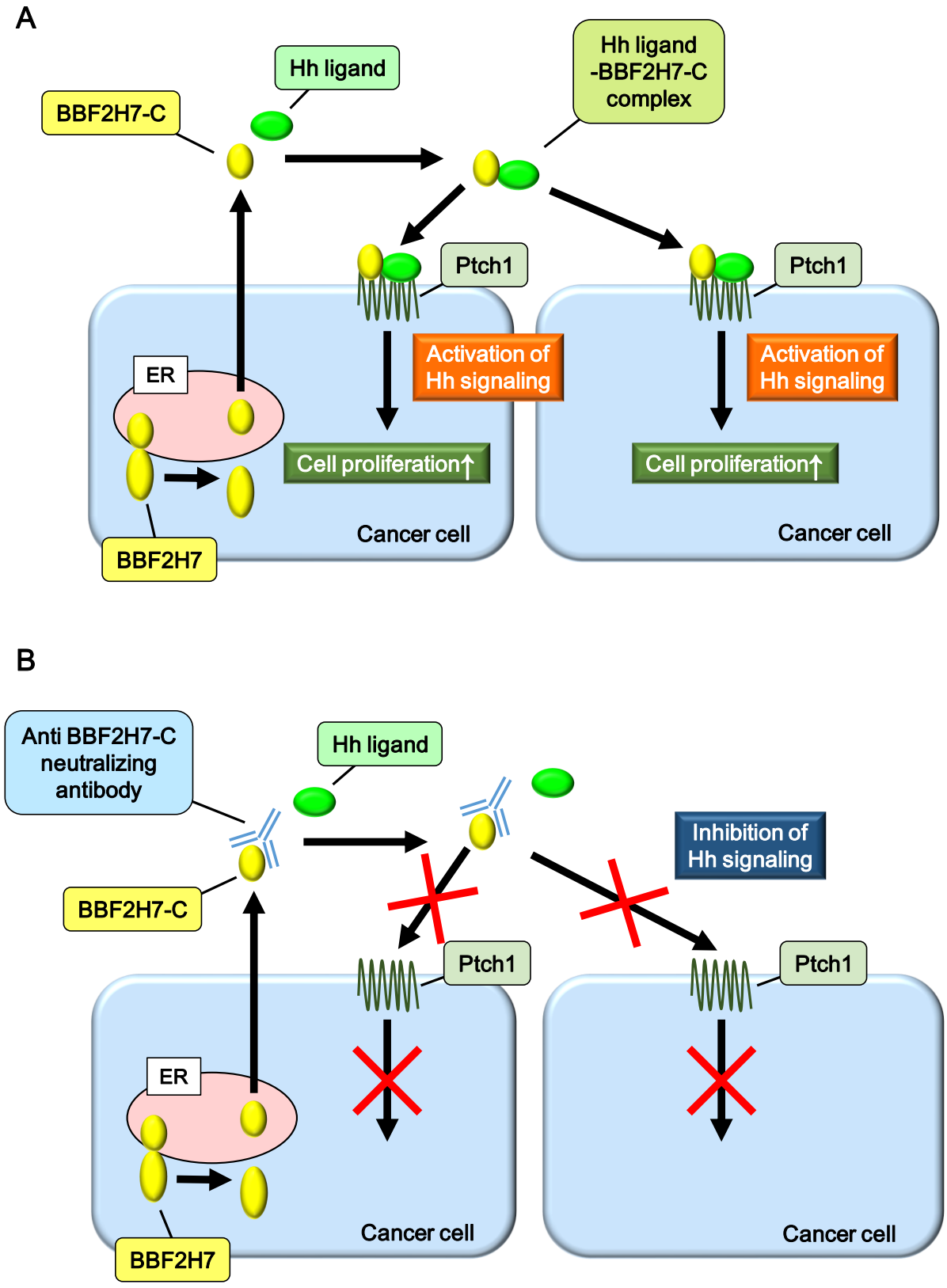

Figure 3

Figure Legends

Figure 1. Structure, distribution and activation mechanism of BBF2H7

(A) BBF2H7 is an endoplasmic reticulum (ER)-resident transmembrane basic leucine zipper (bZIP) transcription factor containing transcription activation and bZIP domains in the N-terminus.

(B) BBF2H7 full-length is cleaved at the transmembrane region in response to ER stress. The cleaved N-terminus containing transcription activation and bZIP domains translocates into the nucleus to induce the expression of various target genes via the binding to cyclic AMP response element (CRE).

(C) *In situ* hybridization of embryonic day 18.5 mouse femur. *Bbf2h7* mRNA is strongly expressed in proliferating chondrocytes of developing cartilage, but not in hypertrophic chondrocytes and trabecular bone.

S1P; site-1 protease, S2P; site-2 protease, RIP; regulated intramembrane proteolysis.

Figure 2. Function of secreted BBF2H7 C-terminus in developing cartilage

Indian hedgehog (Ihh) released from prehypertrophic chondrocytes is trapped by the secreted BBF2H7 C-terminus (BBF2H7-C). The BBF2H7-Ihh complex is efficiently delivered to the periarticular region, followed by inducing parathyroid hormone-related protein (PTHrP) expression through the binding to Patched-1 (Ptch1). Secreted PTHrP promotes chondrocyte proliferation and inhibits the differentiation from proliferating to hypertrophic chondrocytes in the proliferating zone.

Hh; hedgehog.

Figure 3. Approach using anti-BBF2H7 C-terminus neutralizing antibody for the cancer therapy

(A) BBF2H7 C-terminus secreted from cancer cells binds to Hh ligands to facilitate to form Hh ligand-receptor complexes at the cell surface in an autocrine and paracrine manner. Hh signaling activated by the Hh ligand-C-terminus-receptor complex promotes cancer cell proliferation.

(B) Anti-BBF2H7 C-terminus neutralizing antibody binds to the secreted BBF2H7 C-terminal fragment, and inhibits the binding among the C-terminus, Hh ligands and Ptch1. The C-terminus bound by the neutralizing antibody is unable to form the ligand-receptor complex, resulting in inhibiting cancer cell proliferation mediated by Hh signaling.

